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RESEARCH ARTICLE

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The anti-planktonic and anti-biofilm formation activity of Iranian pomegranate peel hydro-extract against *Staphylococcus aureus*

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ABSTRACT

Staphylococcal infections and contaminations have elicited a growing and perennial concern in the medical and food industries. Meanwhile, the manifestation of antibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) beside the production of disinfectant-resistant biofilms makes the confrontation with the bacteria more cumbersome and challenging. Pomegranate peel as a waste product of juicing factories is a natural antibacterial agent. The pomegranate peel hydro-extract (PPHE), as a bio-friendly material, was prepared from an Iranian pomegranate cultivar, Rabab, and its phenolic compounds and antioxidant (*via* DPPH and FRAP assays) and anti-staphylococcal (anti-planktonic and anti-biofilm) properties were assessed. The Rabab PPHE inhibited planktonic cells and biofilm formation of three *S. aureus*. The Rabab PPHE produced large and obvious staphylococcal inhibition zones in which their diameters were significantly dose-dependent for the milk isolated *S. aureus* ($p < 0.05$). Despite the resistance of MRSA (ATCC 33591) to beta-lactam antibiotics, the minimum inhibitory concentration (MIC) of PPHE against its planktonic cells was only 3.75mg mL^{-1}. Furthermore, Rabab PPHE inhibited bacterial biofilms formation in a dose-dependent manner. The MIC of Rabab PPHE against planktonic milk-isolated *S. aureus, S. aureus* (ATCC 29737), and MRSA prevented 47, 36, and 26% of their biofilm formation, respectively. This addresses the differences between the anti-planktonic and anti-biofilm activity of Rabab PPHE. The anti-planktonic and to a lesser extent the anti-biofilm forming activity of this water-based extract supports the notion of its effectiveness and salubrious application in food and pharmaceutical industries.

Keywords

Pomegranate peel, Rabab, Staphylococcus aureus

Abbreviations

PPHE: pomegranate peel hydro-extract
MRSA: methicillin-resistant *Staphylococcus aureus*
MIC: minimum inhibitory concentration
MBC: minimum bactericidal concentration
DPPH: 2,2-diphenyl-1-picrylhydrazyl
FRAP: ferric reducing ability of plasma
BHT: butylated hydroxytoluene

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Introduction

The habitat of *S. aureus*, as a member of the *Micrococcaceae*, is nasopharynx and the hair and skin of more than 50% of healthy people. *Staphylococcus aureus* is the leading cause of staphylococcal food poisoning and extra-intestinal infections. *Staphylococcus aureus* produces many enzymes and toxins that sustain the bacterium and make it resistant to drugs. The enterotoxin of the bacterium is heat resistant, and therefore ordinary cooking, pasteurization, and drying do not easily destroy it (1). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a beta-lactam antibiotics resistant bacterium (2). This bacterium was reported in humans and livestock (mastitic cattle milk) since 1961 and 1972, respectively (3). Antibiotic resistance among pathogens is a worldwide growing problem (4). In 2004, 59.5% of US health centers reported at least one case of MRSA (5). This bacterium has a methicillin resistance gene (*mec-A*). Strains that have this gene also resist many other antibiotics. The resistance makes it difficult to fight them and eventually leads to their further dissemination (6). Staphylococci can form structures called biofilms that attach different surfaces (7). The antibiotic-resistant sessile biofilm forms can further resist the host immune system or be a reason for food contamination and spoilage in the food industry (8-11).

Pomegranate (*Punica granatum* L.) is a native plant of Iran and its neighboring countries which its cultivars have various characteristics (12-14). Rabab pomegranate cultivar which has a thick peel is one of the largest commercial products in the Persian fruit industry (15-17). Pomegranate is classified as a medicinal plant because of its valuable functional compounds (12). Many of its phenolic compounds have drastic antibacterial and antioxidant properties (18-20). The pertinent application of water as a solvent for bioactive compounds extraction from pomegranate peel may provide a safe and relevant extract for the food and pharmaceutical industry which is missed in many evaluations. Until now, studies did not show cytotoxicity towards the by-products of the pomegranate juice industry at arbitrary concentrations (20). Presumably, the staphylococcal complications usually come from both the biofilm and planktonic forms. Lots of the antibacterial agent studies lack a co-assessment of the anti-planktonic and anti-biofilm activity of compounds (20-23). This study aims to assess the antioxidant, total phenolic, and antibacterial effects of an Iranian (Rabab) pomegranate hydro-extract against the planktonic and sessile life of *S. aureus*.

Results

The Rabab PPHE had pale pink to red color. The total phenolic evaluation of the extract revealed that Rabab PPHE has considerable phenolic compounds. Although the Rabab PPHE showed lower antioxidant activity than butylated hydroxytoluene (BHT; synthetic antioxidant), its antioxidant properties in either DPPH or FRAP assays were quite astonishing (Table 1).

Figure 1 shows the staphylococcal inhibition zones produced by Rabab PPHE. The illustrated inhibition zones were quite distinctive in which there was not any tiny colony within their radius. The formation of opaque-milky aura around the dug-wells was probably due to the effect of tannins and astringent compounds of the PPHE on the proteins of Mueller-Hinton agar medium (24).

By increasing the concentration of extract in agar-dug wells the staphylococcal inhibition zones enlarged (Figure 1 and Figure 2). The lowest Rabab PPHE concentration (6mg/well) showed a significantly lower antibacterial activity than other concentrations towards the milk-isolated *S. aureus* ($p < 0.05$). Intriguingly, the higher concentrations (12 and 24mg/well) did not exhibit a significant difference in their antibacterial activity ($p > 0.05$). Statistically, the inhibition zones produced by Rabab PPHE were 6mg/well < 12mg/well = 24mg/well. Notably, the inhibition zones of these three concentrations were not significantly different neither on MRSA nor *S. aureus* (ATCC 29737) ($p > 0.05$).

The minimum bactericidal concentration (MBC) of PPHE was always higher than the MIC (Table 2).

Table 1
The Antioxidant activity (determined by FRAP and DPPH assays) and total phenolic content of Rabab PPHE.

Extract type	DPPH (IC50, mg mL^{-1})	FRAP (mmol Fe(II) g^{-1})	Total phenols (mg GAE g^{-1})
Rabab PPHE	1.13	0.84	143
BHT (positive control)	0.019	1.38	ND

ND: not determined
PPHE: pomegranate peel hydro-extract
BHT: butylated hydroxytoluene
DPPH: 2,2-diphenyl-1-picrylhydrazyl assay
FRAP: Ferric reducing ability of plasma assay

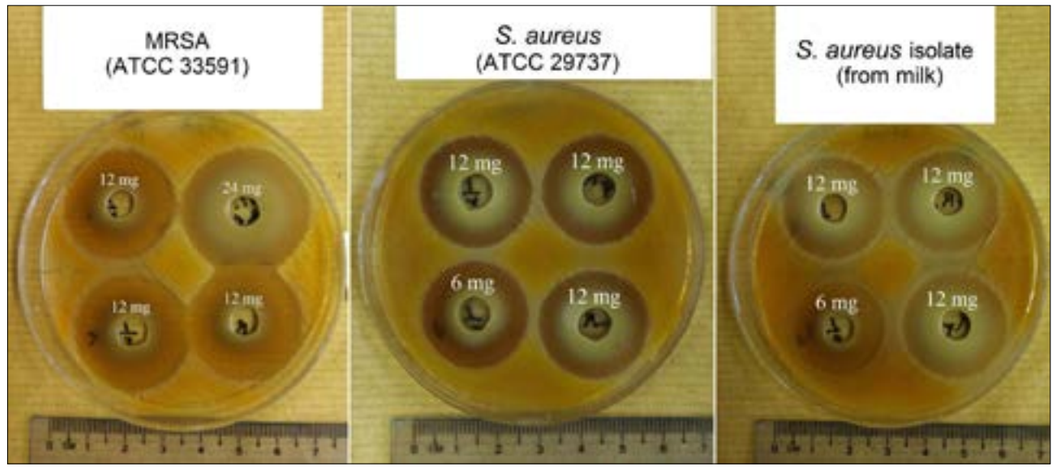

Figure 1.
Antibacterial activity of various concentrations of Rabab PPHE against *S. aureus* by agar well diffusion technique.

Table 2
The MIC and MBC (mg mL^{-1}) of Rabab PPHE against different *S. aureus*.

Antibacterial agent	*S. aureus* (ATCC 29737)		MRSA (ATCC 33591)		*S. aureus* isolate (from milk)	
	MIC	MBC	MIC	MBC	MIC	MBC
Rabab PPHE	15	30	3.75	15	15	60 <
Cefixime (positive control)	8	12	265 <	265 <	8	24

MIC: minimum inhibitory concentration
MBC: minimum bactericidal concentration
PPHE: pomegranate peel hydro extract
MRSA: methicillin-resistant *Staphylococcus aureus*

The MIC and MBC of PPHE against MRSA were lower than other staphylococci. The lower MIC value of the extract towards MRSA represents its strong anti-planktonic activity. In contrast, the MRSA sensitivity to cefixime antibiotic (positive control) was lower than that of the rest of the bacteria. This antibiotic had a significant inhibitory effect on the other staphylococci that was illustrated by lower MIC and MBC values.

The Rabab PPHE inhibited MRSA biofilm formation by 2.8% at low concentration (0.5mg mL^{-1}), whereas the *S. aureus* (ATCC 29737) biofilm was more sensitive than the MRSA to this concentration of Rabab PPHE. By increasing the concentration of PPHE, the anti-staphylococcal biofilm formation activity was also increased (Figure 3). The extract inhibited the biofilm formation of milk-isolated *S. aureus* at intermediate concentrations (from 1.9 to 30mg mL^{-1}) more than other *S. aureus* strains. This extract at 30mg mL^{-1} inhibited more than 50% of the milk-isolated *S.*

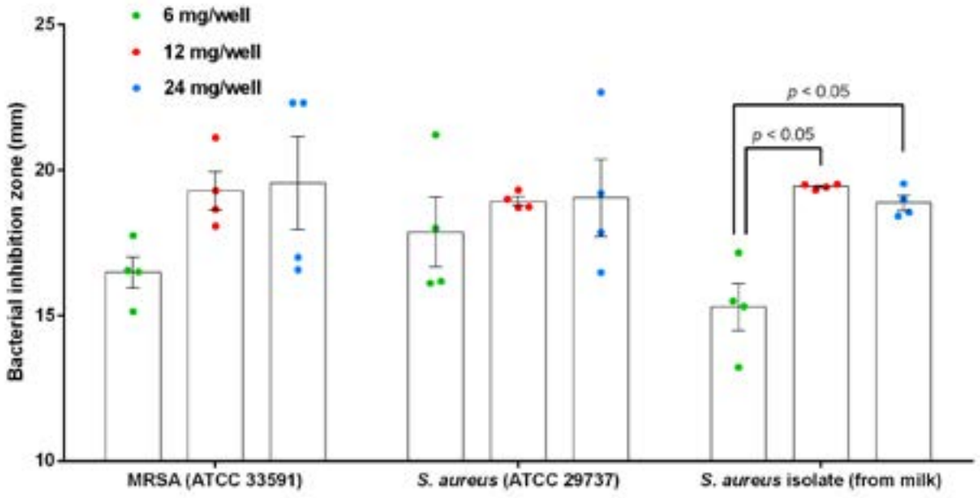

Figure 2.
The effect of three concentrations of Rabab PPHE against *S. aureus* (n=4; mean ± SEM).

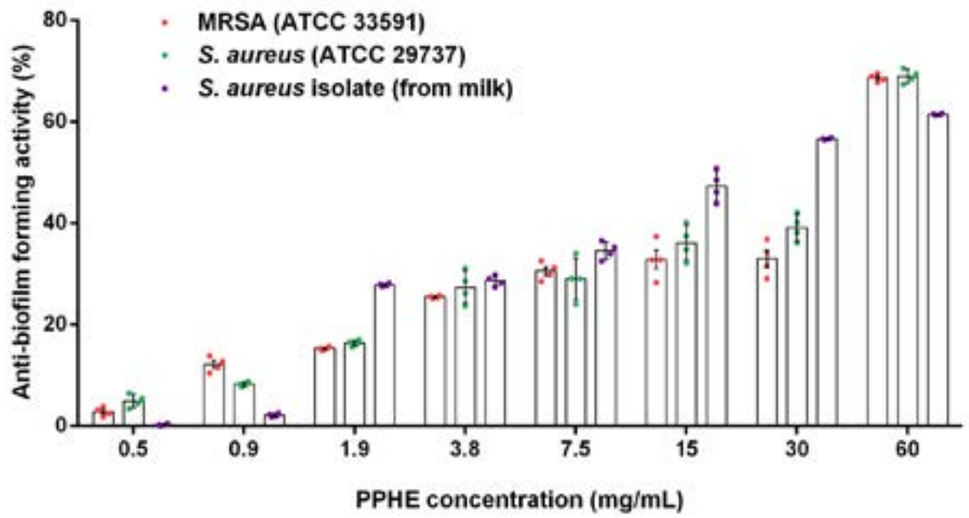

Figure 3.
The anti-biofilm forming activity of different concentrations of Rabab PPHE (n=4).

aureus biofilms.

The anti-biofilm forming activity of the extract was increased profoundly from 30 to 60mg mL^{-1} for MRSA and *S. aureus* (ATCC 29737). Accordingly, Rabab PPHE at 30mg mL^{-1} was not sufficient to inhibit half of the MRSA and *S. aureus* (ATCC 29737) biofilms, while the concentration of 60mg mL^{-1} prevented more than 65% of the biofilm formation by these strains. The milk-isolated *S. aureus* lost 25.7% of its biofilm formation ability while increasing the PPHE exposure from 0.9 to 1.9mg mL^{-1}.

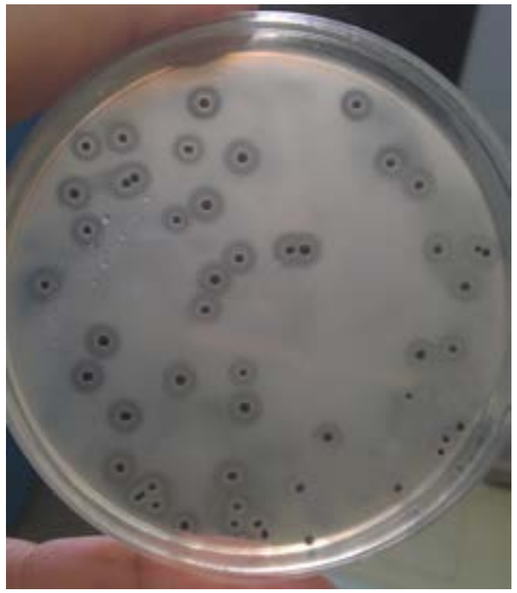

Figure 4.
The growth of *Staphylococcus* colonies with dull haloes on Baird-Parker medium.

Discussion

The extract showed significant anti-staphylococcal and antioxidant activity in this study. It has been shown that the non-edible parts of the pomegranate have higher bioactivity (25). Various pomegranate peel metabolites are very complex (26). Rabab PPHE contains phenolic compounds (Table 1). Many phenolic compounds and organic acids such as gallic acid, chlorogenic acid, caffeic acid, vanillic acid, *p*-coumaric acid, ellagic acid, malic acid, quinic acid, illogic acid, tannins, punicalin, punicalagin, grantin B, casuarinin, corilagin, methyl gallate, kaempferol, catechol, catechin, epicatechin, epigallocatechin 3-gallate, quercetin, rutin, pelargonidin, naringin, and luteolin, have been detected in pomegranate peel (27-30). Plant polyphenolic compounds have antimicrobial and antioxidant effects. In many studies, pomegranate peel alcoholic extracts demonstrated antimicrobial activity (21, 26, 31). Bioactive effects of pomegranate are due to various and abundant bioactive compounds such as tannins (especially ellagitannin, as a hydrolyzable tannin or prodelphinidin, as a condensed tannin). Two members of ellagitannins (namely ellagic acid and punicalagin) play a significant role in the antimicrobial and antioxidant effects (32, 33). The Precipitation of cell membrane proteins by pomegranate peel phenolic compounds causes bacterial cell membrane leakage and ultimately results in cell lysis and death (25, 26). The toxicity of phenolic compounds against bacteria can also occur when they react with thiol groups of proteins that finally prevents the growth of the microorganism (25). Pomegranate peel extracts (esp. alcoholic) exhibited other deleterious effects on bacteria such as inactivating their enzymes or preventing their protein e.g., staphylococcal enterotoxin A (SEA) production (18, 32).

The concentration of the bioactive compounds in the pomegranate depends on the pomegranate cultivar and the different stages of plant growth (25, 26). Furthermore, the antibacterial and antioxidant activity of pomegranate extract depends on the plant cultivar and geographical origin, harvesting season, and extraction method (34). For example, the hydro-extracts of South Africa or Yemen pomegranate cultivars in previous studies, contrary to the current study, did not show antibacterial activity against *S. aureus* (26, 35). The total phenolics and antioxidant capacity (evaluated by DPPH assay) of Rabab PPHE in this study were less than the methanolic extracts of other pomegranate cultivars in the Fawole et al. (2012) study (26). These differences are probably related to the characteristics of the pomegranate cultivars and the type of fruit peel extract. The extraction method plays an important role in the quality of the extract. It has been stated that the extraction of polyphenolic compounds depends on the type of solvent, plant particle size, solvent to plant solid ratio, and extraction temperature and time. Extract preparation with high temperatures for long times may reduce the level of polyphenols, including ellagitannin (36). Application of hot water or soxhlet extractor to obtain the extract and the autoclave-sterilization probably reduce its antibacterial activity (22, 23).

Pomegranate polyphenols are considerably extracted with hydrophilic solvents. The hydro-extracts lack the toxicity of solvent remnants and therefore can be stored wet while retaining high antioxidant activity (25). Furthermore, hydro-extracts are more compatible with the hydro-nature of the body cells and probably make better systemic effects. The nature of PPHE makes it a highly soluble and diffusible extract in the Muller-Hinton Agar medium. This extract has potent anti-staphylococcal effects, and the combination of this effect along with its facile diffusion generated significant bacterial inhibition zones even at low concentrations (Figure 1). The bacterial inhibition zones did not show any significant difference between the sensitivity of the three staphylococci exposing PPHE ($p > 0.05$).

The planktonic MRSA cells showed a significant sensitivity to the extract in MIC assay. Even the MBC of PPHE against this bacterial strain was lower than other staphylococci (Table 2). The MRSA infections are mainly divided into two hospitals acquired (HA-MRSA) and community-acquired (CA-MRSA) groups (4). Despite hospital infections, the prevalence of MRSA is higher in patients with open ulcers and immune deficiency (37). The sensitivity of MRSA to PPHE is very important due to the resistance of this bacterium to β-lactam antibiotics such as cefixime as a third-generation broad spectrum cephem (Table 2). Methicillin and beta-lactam interfere with bacterial cell wall peptidoglycan by binding the penicillin-binding proteins (PBPs). Nevertheless, MRSA resists β-lactam antibiotics by producing PBP2a instead of PBPs due to the acquisition of the *mec-A* gene (4).

The staphylococcal biofilm formation was also strongly influenced by different concentrations of PPHE. The Rabab PPHE at a concentration of 60mg mL^{-1} prevented nearly 70% of the staphylococcal biofilm formation. The anti-biofilm formation activity of PPHE was dose-dependent and elevated by increasing the extract concentrations. The extract at 1.9mg mL^{-1} made a sharp slope in inhibiting the milk-isolated *S. aureus* biofilm formation. However, this sudden increase in the inhibition of MRSA and *S. aureus* (ATCC 29737) biofilm formation occurred at 60mg mL^{-1} (Figure 3).

The extract at its MIC (15mg mL^{-1}) for the milk-isolated *S. aureus* and *S. aureus* (ATCC 29737) inhibited 47% and 36% of their biofilm formation, respectively. Moreover, 26% of the MRSA biofilm formation was inhibited by the MIC of the extract against this strain (i.e. 3.75mg mL^{-1}). Therefore, the MIC of PPHE against planktonic MRSA shows lower anti-MRSA biofilm formation activity regarding other staphylococci. Notably, the PPHE at the lowest concentration (0.5mg mL^{-1}) inhibited albeit a low percentage (2.8%) but substantial anti-MRSA biofilm formation activity (Figure 3). Hence, concentrations below the MIC also show anti-biofilm forming activity. Plausibly, inhibition of biofilm formation by the extract is achieved not only through bacterial growth inhibition but also through other mechanisms. The exact mechanism for the biofilm formation inhibitory activity of PPHE is still shrouded in mystery. There are some conjectures about especially the alcoholic extracts (32, 38-40). The effect of these extracts on biofilms is probably due to their ellagic acid. Pomegranate extract can precipitate proteins, such as adhesins, which are crucial in biofilm formation. Moreover, tannins such as ellagic acid alter the surface charge of bacteria and subsequently may interfere with the cell-substratum attachment. The pomegranate extract can also disrupt the pre-formed biofilms of various bacteria (32). It has been established that the hydro-alcoholic extract of pomegranate rind shows a good anti-quorum sensing activity. Quorum sensing is a kind of bacterial communication that biofilm formation interconnects with it (38).

In conclusion, Rabab PPHE shows remarkable anti-staphylococcal effects. The extract has high levels of phenolic compounds. Antioxidant and antibacterial effects of PPHE are probably due to its bioactive compounds such as tannins (e.g., ellagitannin or prodelphinidin). The application of this extract will be useful in inhibiting and eliminating staphylococcal food contaminations or body infections. These antibacterial effects against MRSA are of great importance. The PPHE showed lower anti-biofilm formation activity than the anti-planktonic activity against staphylococci. This was more vivid in the case of MRSA. Although inhibition of MRSA biofilm by PPHE begins at low concentrations, the PPHE level equivalent to the MIC has less inhibitory activity against MRSA biofilm formation than other staphylococci. Conversely, the effect of PPHE against some staphylococcal biofilms provokes the use of this water-soluble extract in food and pharmaceutical industries and even milking machines disinfection. However, the efficacy and stability of PPHE and its active ingredients under various industrial processing and simulated body conditions need to be profoundly explored.

Material and methods

Preparation of pomegranate peel hydro-extract (PPHE)

Commercially ripe and fresh pomegranates were harvested during October 2017 from mature trees. The pomegranates were from Rabab-e-Neyriz (from Fars Province) cultivar as a known Persian pomegranate cultivar. The pomegranate was authenticated by the Department of Plant Productions, Agricultural Faculty of Bardsir, Shahid Bahonar University of Kerman. Fifty pomegranates were collected and flushed by tap water and then washed

three times with distilled water. After drying, the pomegranates were peeled while the peel and pulp compartments were carefully separated from each other. The pomegranate peels were shadow dried for 7 days and then grounded with a grinder. The Rabab peel powder mixed with distilled water (0.2g mL^{-1}) and the homogenate was agitated gently at 25 °C for 24h in a shaking incubator (JSSI-100C Compact shaking incubator, JSR). The suspension was centrifugated at 3000 rpm for 30 min at 4 °C using Universal 320R centrifuge (Hettich, Tuttlingen, Germany). The supernatant filtered through a filter paper (Whatman No. 1) and after that filter-sterilized with 0.22µm filters (Millipore Sigma, Millex®-GV). The filtrates were lyophilized and stored at 4 °C as the hydro-extract (41). The anti-staphylococcal activity of the extract was assessed in less than seven days while being filter-sterilized before application.

Total phenolic of PPHE

The total phenolic content of PPHE was determined spectrophotometrically by the Folin-Ciocalteu method (42, 43). A 0.5ml aliquot of diluted PPHE was mixed with 0.5ml of 10-fold-diluted Folin-Ciocalteu's reagent. After 5 minutes of shaking, 0.5ml of sodium carbonate solution (20%) was added. Ultimately, the solution was brought up to 5ml by distilled water and incubated at 25 °C for 90 min in the dark. The absorbance of the mixture was measured at 765 nm against a blank (Shimatzo, Japan, UV-1201). The total phenol content obtained using gallic acid as a standard phenolic acid. The PPHE total phenolic content was expressed as mg gallic acid equivalents (GAE) per gram PPHE.

Ferric reducing/antioxidant power (FRAP) of PPHE

Aliquots of iron (III) chloride solution (20 mM), 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ; 10 mM in 40 mM HCl) and acetate buffer (pH 3.6; 300 mM) mixed in proportions of 1:1:10 (v/v), respectively to produce the FRAP reagent. Thence, 100 µL of filtered PPHE were added to 3.0 ml of the 37 °C warmed up FRAP reagent. The Absorbance (593 nm) was recorded after 5 min. Similarly, the standard curve was prepared using iron (II) sulfate solution and butylated hydroxytoluene (BHT) used as a positive control. The antioxidant capacity was expressed as mmol of Fe (II) per g extract (44).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay of PPHE

This assay is based on the ability of antioxidants to decolorize DPPH, a stable free radical. Briefly, the PPHE was diluted in methanol, and 50 µl of each dilution was mixed with 2.5 mL of a fresh DPPH radical methanol solution (0.004%; w/v). The purple mixture allowed to stand for 30 min in the dark at room temperature. The Absorbance (517 nm) were recorded on a spectrophotometer (Shimatzo, Japan, UV-1201) using methanol as a blank. The radical inhibitory activity of PPHE was calculated as follows:

% inhibition = [(A blank – A sample)/A blank] × 100

Where; "A blank" is the absorbance of the control and "A sample" is an absorbance produced by the extract. Extract concentration providing 50% inhibition (IC50) was calculated from the radical inhibition vs PPHE concentration graph. BHT was used as a positive control (45).

Isolation and identification of S. aureus from milk

During the summer season, cow milk samples were collected aseptically from local farms and transferred on ice to the laboratory. Samples were serially diluted in sterilized normal-peptone (0.85% and 0.1%) and 100 µL of each diluted sample were surface plated onto Baird-Parker agar (M043, HiMedia, India) supplemented with egg yolk and potassium tellurite and incubated aerobically for 48h at 37 °C. The black colonies surrounded by an opaque and also clear haloes were considered as staphylococci (Figure 4). The milk isolates were assessed by Gram-staining, catalase, and coagulase tests until finding the intended bacteria. The gram-positive cocci with positive coagulase and catalase results were further identified as *S. aureus* by API Staph system (BioMe´rieux, 20500, France) (46).

The anti-staphylococcal activity of PPHE

The antibacterial activities of PPHE against *S. aureus* (ATCC 29737), Methicillin-resistant *S. aureus* (MRSA) (ATCC 33591) and the milk-isolated *S. aureus* were assessed by agar-well diffusion method. The MIC of PPHE against the planktonic bacteria was assessed via a micro-broth dilution technique. The MBC of PPHE was further assessed. Finally, the ability of PPHE in the prevention of staphylococcal biofilm formation was measured by a microtiter plate test.

Agar well diffusion assay

The anti-staphylococcal activity of the Rabab PPHE was determined by the agar well diffusion method with some modifications (47, 48). The aforementioned *S. aureus* bacteria were inoculated on the cation-adjusted Mueller-Hinton II broth (90922, Fluka) at 37 °C for 18h. The bacterial suspension density was adjusted equal to that of the 0.5 McFarland standard. The density standardized bacterial culture was swabbed on the solidified Mueller-Hinton agar (70191, Merck) and allowed to dry for 10 min. Thence, 6 mm-diameter wells were made with a sterilized cork-borer in the inoculated Mueller-Hinton agar plates. The lower part of wells was first sealed with a few drops of molten agar medium (49). 100 µL of the PPHE (6, 12, and 24mg per well) were added into the wells and allowed to diffuse at room temperature for 15 min. Negative (sterilized distilled water) and positive controls (Gentamicin sulfate salt, G1264 sigma; 10 µg $well^{-1}$) were also placed in wells. The plates incubated at 37° C for 16-18h. The anti-staphylococcal activity of the extract revealed by the formation of bacterial inhibition zones around the wells and the diameter of the halos were measured by a caliper.

Determination of MIC and MBC of PPHE

The MIC of PPHE against the planktonic bacteria was assessed using a 96-well sterile microtiter plate as described before via a micro-broth dilution technique (50). Briefly, the MICs were evaluated after providing 2-fold dilutions of the extract (60 to 0.47mg mL^{-1}) with cation-adjusted Mueller-Hinton II broth. The overnight bacterial suspensions were diluted and added into the wells to provide the final inoculum of 5×10^5 CFU mL^{-1}. After incubation (37 °C, 24h), optical densities (OD620) of the extract exposed bacteria were studied relative to the negative control. The bacterial growth inhibition was calculated as follows:

% inhibition = 100 – [(OD620E – OD620B) / (OD620G – OD620B)] × 100

Where; "OD620E", "OD620B", and "OD620G" are the optical densities at 620 nm for extract containing wells, background control wells, and growth control wells, respectively. The MIC was the lowest concentration of PPHE that completely (100%) inhibited bacterial growth. After the MICs were read and recorded, the 96-well MIC plates were shaken and re-incubated for an extra 4h at 37°C. Subsequently, the bacteria of wells with no visible bacterial growth were enumerated using Trypticase soy agar (22091, Merck) at 37 °C/24h. The minimum bactericidal concentration was defined as the lowest concentration of PPHE that causes ≥99.9%

staphylococcal kill relative to the first inoculum. Cefixime trihydrate (18588, Fluka) was used as a positive control.

The anti-biofilm forming activity of PPHE

To evaluate the effect of PPHE against staphylococcal biofilm formation, a microtiter plate adhesion assay was applied (51). In a 96-well plate, the PPHE was serially diluted with cation-adjusted Mueller-Hinton II broth from 60 to 0.47 mg mL^{-1} in a 2-fold manner. Thence, 100 µL of diluted overnight staphylococcal suspension (1:100) was added to each well. The microtiter plate was incubated at 37 °C for 24h to let the bacteria form different levels of biofilm. After incubation, 200 µL of crystal violet (0.06%, w/v) was added to each well and the plate was shaken three times to help the biofilms stain. After 15 minutes at 25 °C, each well was washed at least three times with sterile normal saline (200 µL) to remove planktonic cells and the unfixed stain. The biofilm-bound crystal violet was further extracted with 200 µL of ethyl alcohol (95%) and transferred to a 96-well plate. The absorbance (595 nm) was recorded by a microplate reader to determine the level of biofilm formation. Culture medium and also different concentrations of the extract without the bacteria were used as the control. The inhibitory activity of PPHE on the staphylococcal biofilm formation was evaluated by comparing the ODs of the treatments with negative controls.

Statistical analysis

Anti-staphylococcal inhibition zones and anti-biofilm forming activities were represented as mean ± SEM of the results in quadruplicates. Data analysis was carried out using SPSS software (SPSS, Chicago, Ill., USA). One-Way ANOVA followed by Duncan's post hoc test (alpha = 0.05) was used to analyze the differences of inhibition zones between staphylococci and also the levels of PPHE.

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Author Contributions

H.E. conceived, designed, and analyzed the data. H.E. and M.E. performed the experiments and wrote the paper. L.M. provided some of the materials.

Conflict of Interest

The authors declare that there is no conflict of interest.s.

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